MACSQuant Tyto Cell Sorter

FCCSF TRAINING

DOCUMENT

2024-08-05

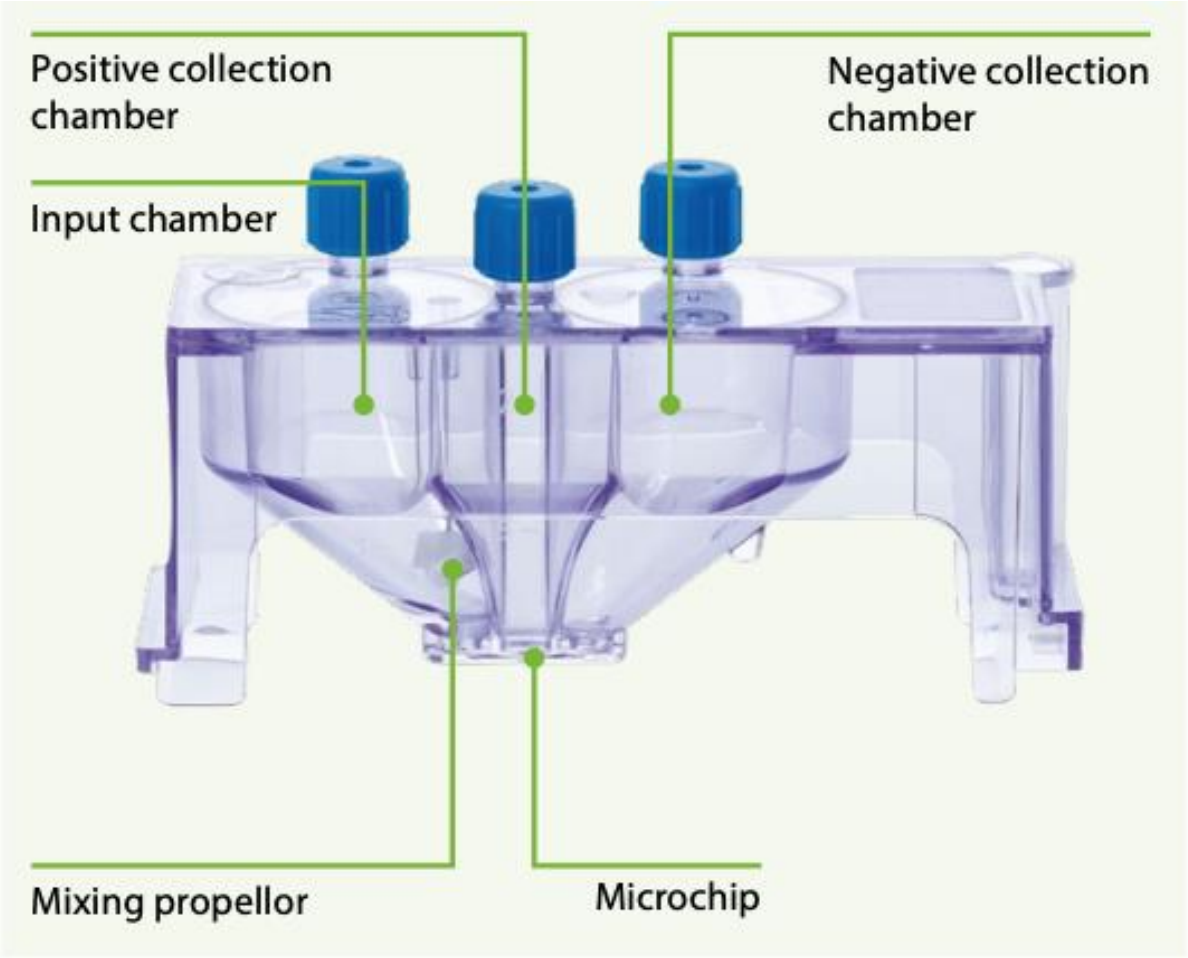
Overview: Configuration

- 3 lasers: 405, 488 and 638
- 10 fluorescent detectors plus backscatter blue and backscatter violet
- Note: There is no forward scatter detector and all parameters are height and not area of signal pulse

	Filter	Channel	Miltenyi Biotec dyes	Other dyes	
Violet 405 nm	450/50 nm	V1	**VioBlue®** **Viobility™ 405/452 Fixable Dye** **DAPI** **Hoechst 33342**	Alexa Fluor® 405 BD™ Horizon™ V450 BV421™ Calcein Violet 450 AM Cascade Blue® CFP	eBFP eFluor® 450 Hoechst Dyes Pacific Blue™ Vybrant® DyeCycle™ Violet Zombie Violet™
	525/50 nm	V2	**VioGreen™** **Viobility™ 405/520 Fixable Dye**	Alexa Fluor® 430 AmCyan BD™ Horizon™ V500 BV510™ Cascade Yellow™	Krome Orange™ Pacific Orange™ Qdot® 525 Zombie Aqua™
Blue 488 nm	525/50 nm	B1	**FITC** **VioBright™ FITC** **Vio® 515** **VioBright™ 515** **Viobility™ 488/520 Fixable Dye**	Alexa Fluor® 488 Calcein AM DyLight® 488 CFSE GFP	SYTOX® Green Vybrant® DyeCycle™ Green YFP Zombie Green™
	585/40 nm	B2	**PE**	Cy™3	Vybrant® DyeCycle™ Orange
	655–730 nm	B3	**PerCP** **PerCP-Vio® 700** **PE-Vio® 615** **Propidium Iodide** **7-AAD**	PerCP-Cy™5.5 PE-Cy™5.5 PE-Cy™5 ECD PE-Texas Red®	BD™ Horizon™ PE-CF594 PE-eFluor® 610 PE-Alexa Fluor® 610 PE/Dazzle™ 594 PerCP-eFluor® 710
	750 nm LP	B4	**PE-Vio® 770**	PE-Alexa Fluor® 750	PE-Cy™7
Red 638 nm	655–730 nm	R1	**APC** **Vio 667®** **VioBright™ 667**	Alexa Fluor® 647 Alexa Fluor® 700 APC-Alexa Fluor® 700	Cy™5 DRAQ5™ eFluor® 660
	750 nm LP	R2	**APC-Vio® 770**	APC-Alexa Fluor®750 APC-Cy™7 APC-eFluor® 780	APC-H7 Zombie NIR™

Overview: Cartridge

- Sorting takes place within the enclosed, sterile cartridge
- Fluorescently labeled cells are loaded into the input chamber
- The microchip located at the bottom of the cartridge enables high-speed, fluorescence-based cell sorting
- A mechanical valve redirects cells into either the positive or negative collection chamber



Overview: How it works

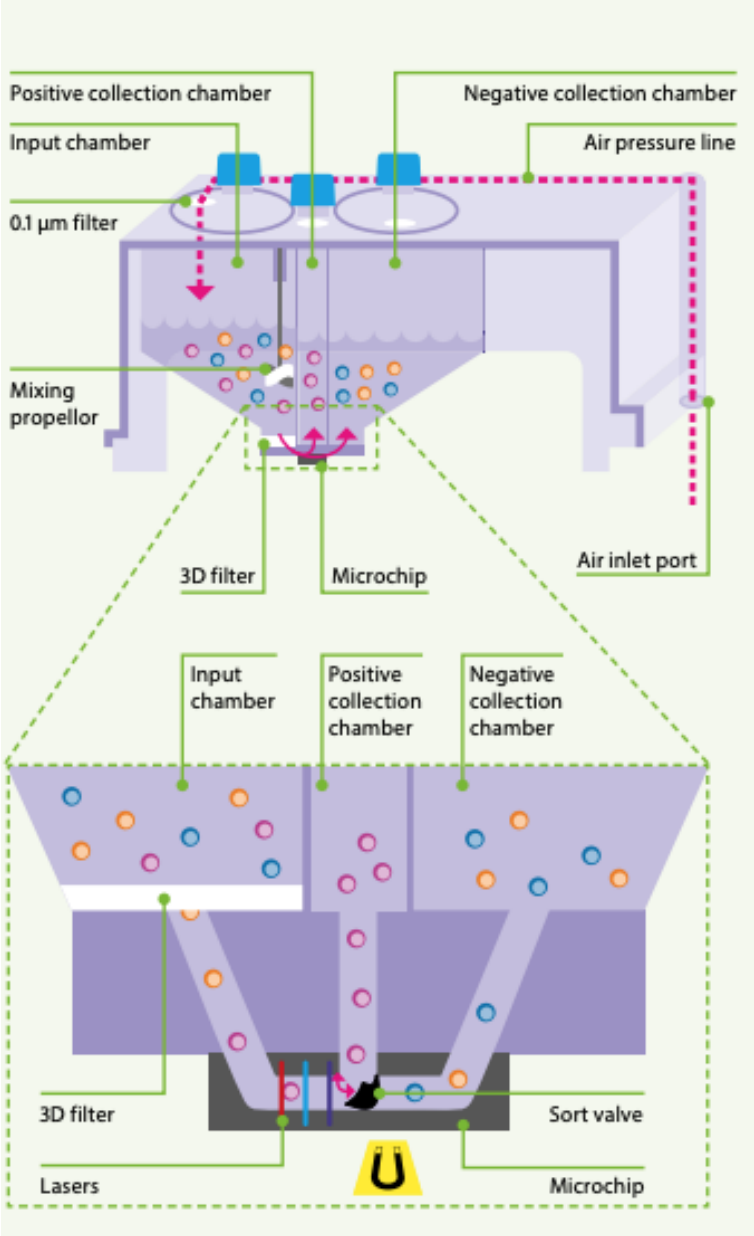
Filtered air enters input chamber through a microchannel.

Low pressure (<3 PSI) drives cells through the microchannel into the microchip where it passes through the lasers.

Cells are identified for sorting by scatter and fluorescence.

A magnetic field is applied to the microchip which triggers the sorting valve to open. The movement of the valve sends the target cell into the positive collection chamber. A silicon spring then returns the valve into its original position.

Short video on how Tyto works



Pre-sort Considerations

Panel design

- If feasible, choose fluors so that compensation is not needed.
  - Select dyes from different lasers
  - Use FluoroFinder Spectral Viewer for Tyto to estimate the amount of overlap

- **Pre-sort analysis**
- Sort purity is in part dictated by your population of interest as a percent of total cells and cell concentration
- Prior to sorting, run your sort sample on one of our flow cytometry analyzers (Fortessa or Aurora) to determine this percentage

- **Pre-sort viability**
- Sort quality is also dependent on concentration
  - Get a count of cells per ml using the Vi-Cell or other cell viability analyzer
  - Using the Purity calculator to predict the max achievable purity
  - Use the concentration calculator to determine cell concentration needed to achieve the desired sort purity

Pre-Sort Calculations-Poisson worksheet

Link to Poisson Calculator Worksheet

How to use the calculator

(SW v1.x)

Purity calculator – to predict the max achievable purity

1. Choose cartridge type: **Regular** (Standard) or **Highspeed** (HS)
2. Enter the total cell concentration of your Input sample
3. Enter the frequency of target cells in your Input sample
4. Type in the Input sample volume in mL (for sort fraction volume prediction)

Concentration calculator – to determine cell concentration needed to achieve a desired purity

1. Enter your desired purity (cell F4) for target cells in the **Sort** fraction
2. Note that target cell frequency (cell C7) must also be filled out first

→ Read out the maximum **permitted cell concentration** for the **Input** sample to reach the desired sort purity

Notes:

- We recommend to always keep the sort rate below 5000 cells/sec
- Flow rate is 4mL/h for the Regular & 8mL/h for Highspeed (HS) cartridge
- Yield & sort efficiency cannot be predicted as they depend on sample quality & handling among other factors

Checklist

- Purchase a cartridge in iLab
- Bring sample to SMACC in concentrated form
- Run on Vi-Cell and determine viability and concentration
- Run sample on flow cytometry analyzer and determine % of cells of interest
- Plug numbers in Poisson calculator to determine max achievable purity and optimal sort sample concentration
- Take your sample to the BSC and dilute to the optimal concentration.
- Prime the cartridge
- Filter through a 20µm filter. If your sample is clumpy, use a buffer with EDTA or DNAse. Tyto Running Buffer has DNAse in it. Do not use DNAse if downstream application is sequencing.
- Note: if your sample clogs the cartridge, the only option is to use another cartridge so make sure it is very high quality
- Note: Only one population can be sorted at a time. You can sort serially to sort multiple populations

Sample prep and supplies needed

- Input and output chambers hold a maximum of 10ml. The sort chamber holds a max volume of 2ml. Your sorted sample will be highly concentrated
- Samples with 80% viability or more will work best. Consider removing dead cells in messy samples
- Filter with 20µM filter is required because the microfluidics channel is 25µM wide. Cells larger than 20µM cannot be sorted.
- Samples can be sorted in media without phenol red and without serum.
- Normal cartridges sort at 4ml/hour
- High speed cartridges sort at 8ml/hour
- Supplies needed
  - Tyto Cartridge
  - MACSQuant Tyto Running Buffer or other media or buffer
  - MACSQuant Priming fixture -located in SMACC
  - 20uM cell strainers
  - 10 ml syringe with Luer-Lock tip
  - Gel Loading pipette tips or sterile extended tip transfer pipettes

Prime Cartridge

NOTE: For sterile sorts, all open cartridge steps should be performed in a biosafety cabinet using sterile technique.

Note: When handling the cartridge, be careful not to touch the chip on the bottom.

1. Place the cartridge into the MACSQuant Tyto Priming fixture by sliding the feet of the cartridge into the corresponding slots of the base.

NOTE: The Priming Fixture has a magnet that holds the valve open allowing fluid to enter the microfluidic channel through all three chambers.

2. Remove the blue cap from the input chamber (the one with an owl on it.)

3. Using a pipette with gel loading tip or extended tip transfer pipette, add 200-500µL of sort buffer of your choice into the sample input chamber.

Prime Cartridge Cont.

1

Fill syringe with air: Pull the plunger of a 10 mL syringe with male Luer-Lock connection fitting out to its stop. Screw the syringe to the input chamber (one with the owl).

2

Push the plunger while pressing on the the corner of the cartridge with a circle (front, left) to flow the sort buffer to the positive collection chamber.

3

Hold the plunger down until buffer appears in the positive collection (middle) chamber. It will be a very small amount and may be hard to see.

Prime Cartridge continued

5. Remove the cartridge from the Priming Fixture. **Be careful not to touch the microchip when handling the cartridge**
6. Close the pressure inlet with O-ring with your finger. Push the plunger of the syringe completely to flow the buffer to the negative collection chamber. Hold the plunger down until the buffer level is just above the 3D filter.
7. Unscrew the syringe from the cartridge and unhand the pressure inlet with O-ring.

9. Remove the buffer from the input chamber by using the syringe. Reconnect the cap to the input chamber. Remove the buffer from the positive and negative collection chambers by using a capillary pipet tip.

Cartridge Priming and Sample Loading

Sample Filtering and Loading

Note: Sample loading volume should be between 500 µL and 10 mL.

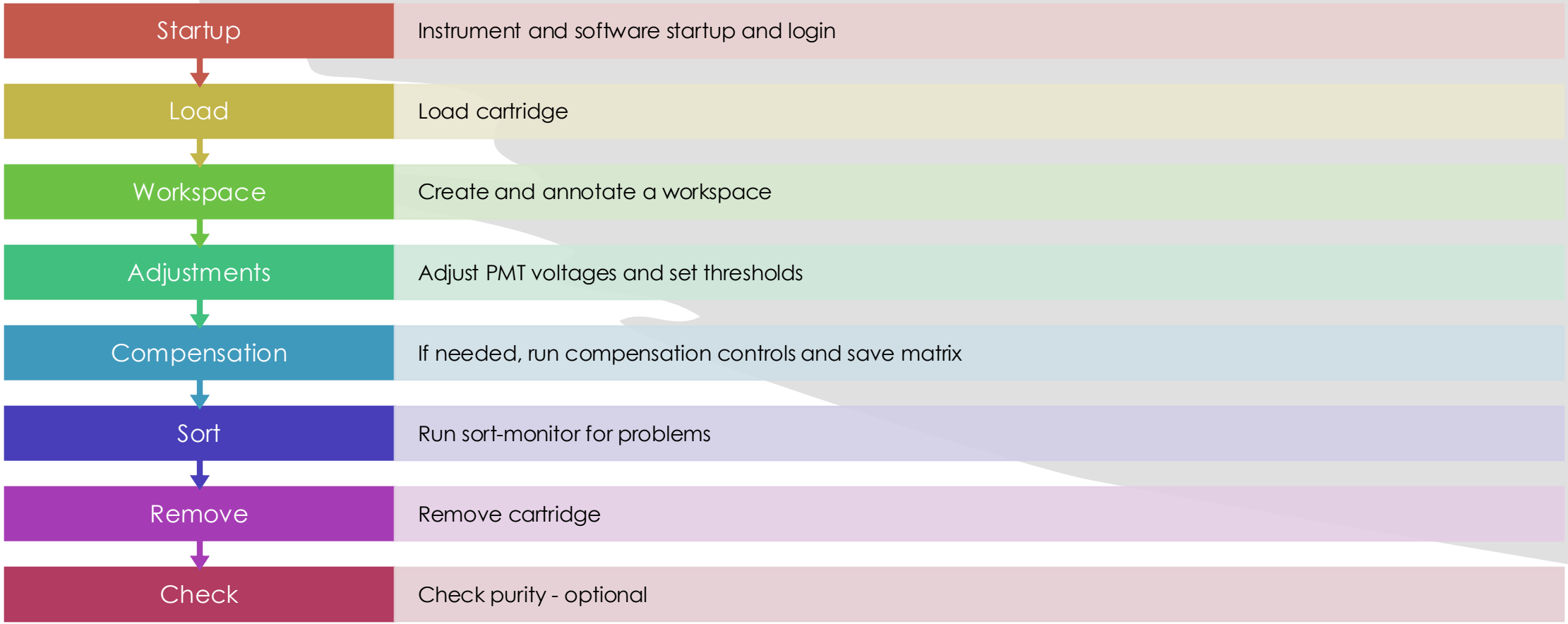
Adjust cell concentration to optimal recommended by Poisson Calculator. Maximum cell density is $5x10^7$ cells/mL.

The syringe should still be attached to your cartridge. If not, reattach syringe and remove the barrel of the syringe.

Place a 20µm filter into the syringe barrel (Miltenyi part# 130-101-812). Apply the cell suspension onto the filter to remove cell clumps. Discard the filter.

Carefully place the plunger on top of the the syringe. Push the sample into the input chamber by applying the plunger at a rate of 0.5 mL/s. A higher rate may force cells into the negative collection chamber. Remove syringe and replace cap.

Sorting Workflow
Reference document: MACSQuant Tyto Short Instructions



Startup

1.1 Instrument startup

1 Ensure that the MACSQuant® Tyto® Instrument main power switch at the rear is in the on-position 'I'.

Note: The system will be in standby mode, indicated by a red LED on the orange touchscreen monitor.

2 Turn on the MACSQuant Tyto Instrument by tapping the orange touchscreen monitor.

1.2 Boot the software

1 Launch the MACSQuantify™ Tyto® Software by double-clicking the MACSQuantify™ Software icon on the desktop.

2 After logging on to the MACSQuantify™ Tyto® Software, wait for the camera on the orange touchscreen monitor to turn on before proceeding. Three crosses (red, blue and violet), indicating the location of the different lasers, and an alignment cross (yellow) will appear (**Figure 1.1**).

Figure 1.1: Camera view on touchscreen monitor

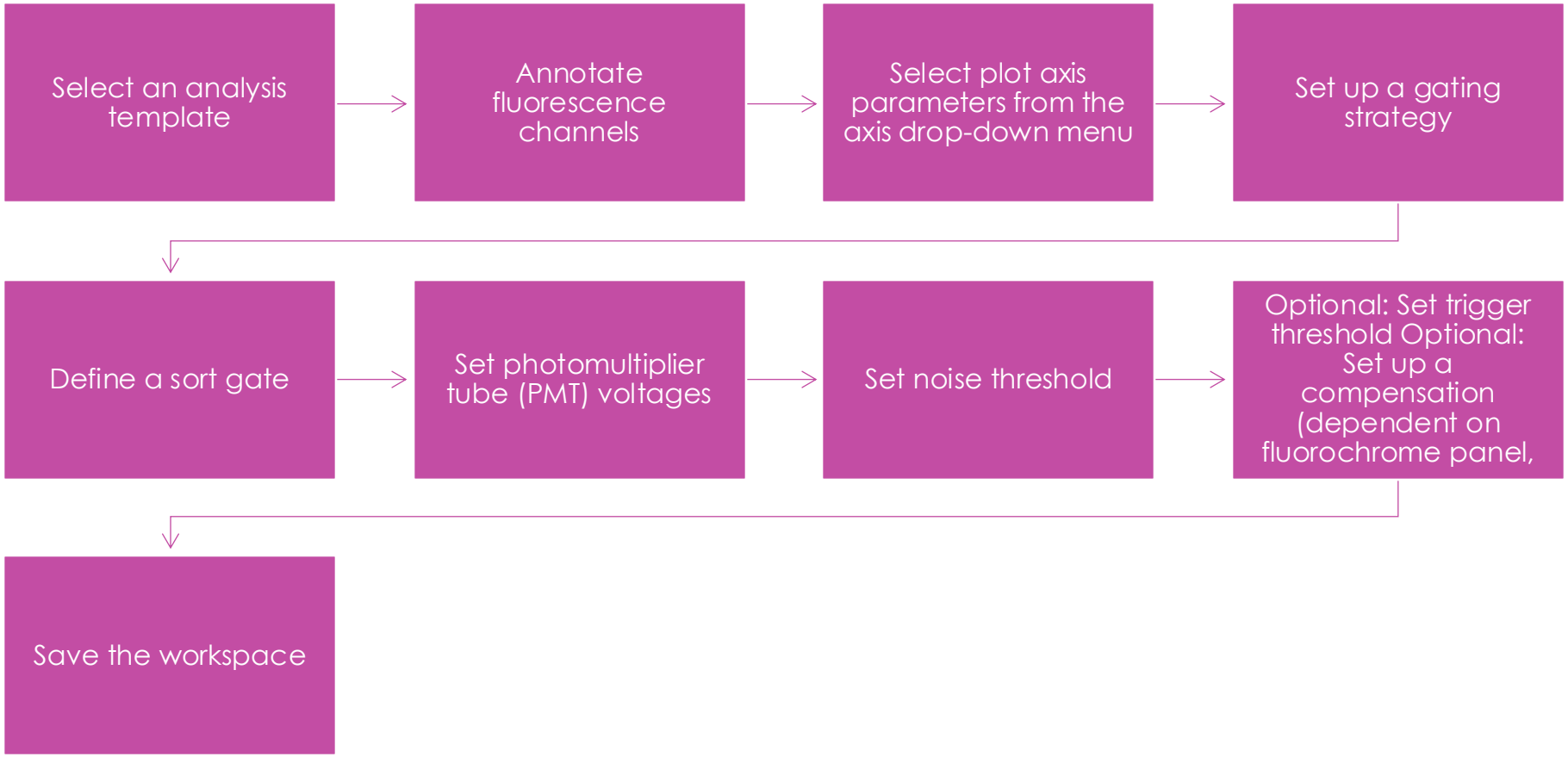
Scan and Load Cartridge

- Scan barcode by holding cartridge in front of code reader. A pop-up window will appear if scanning was successful.
- Load the cartridge:
  - Open the instrument door (Figure 3.1).
  - Orient the cartridge so that the Tyto owl image is in the upright position, nearest to user.
  - Place the cartridge in the right slot of the instrument stage
  - First lower the front side (1.) and then lower the rear side (2.) Do NOT push the cartridge downward. You will hear a click as the cartridge automatically locks into position.
  - The door closes automatically. The handle flashes red and an alarm sounds 10 seconds before closure. Hold the door open if you need more time to insert the cartridge.

Figure 3.1: Correct position of a cartridge.

1	Instrument door
2	Cartridge
3	Tyto owl
4	Slot for a second cartridge

Preliminary Workflow: Setup for Sort



Setting up an experiment

Log in to your user profile and select an experiment.

In the side panel under Experiment tab:

1. Fill in Project and Sample ID
2. Modify the annotations for the fluorescence channels. Enter a blank space for channels not in use
3. Select processing volume (how much volume is in pre-sort chamber) or leave at 10,000µl. The sort will stop at 10ml or when the
4. Set temperature of cooling chamber (4-25C)

Select an analysis window

1 Click on the **New analysis window button** in the toolbar to select a plot layout.

Choose the layout based on the number of fluorophores you are using plus four

Figure 2.1: Predefined templates.

2 From the pop-up window, select a layout suitable for sample analysis. In this example, a 3x2 plot layout was selected (green square in **Figure 2.1**).

Customize Plots-Plot Type

1 Select the desired plot.

2 To change plot types, click the **Information button** near the top right corner of each plot and choose between dot plot, density plot, and histogram under the **View tab** of the dialog that pops up (**Figure 2.3**).

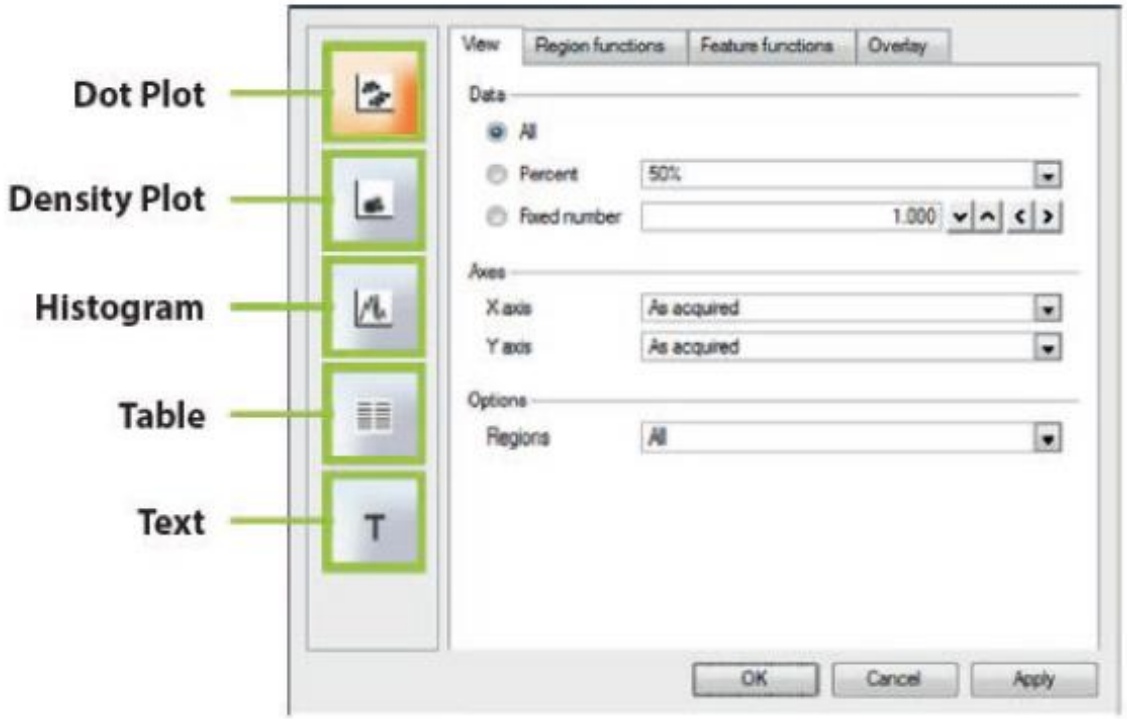


Figure 2.3: Change data display

Customize Plot – change axes

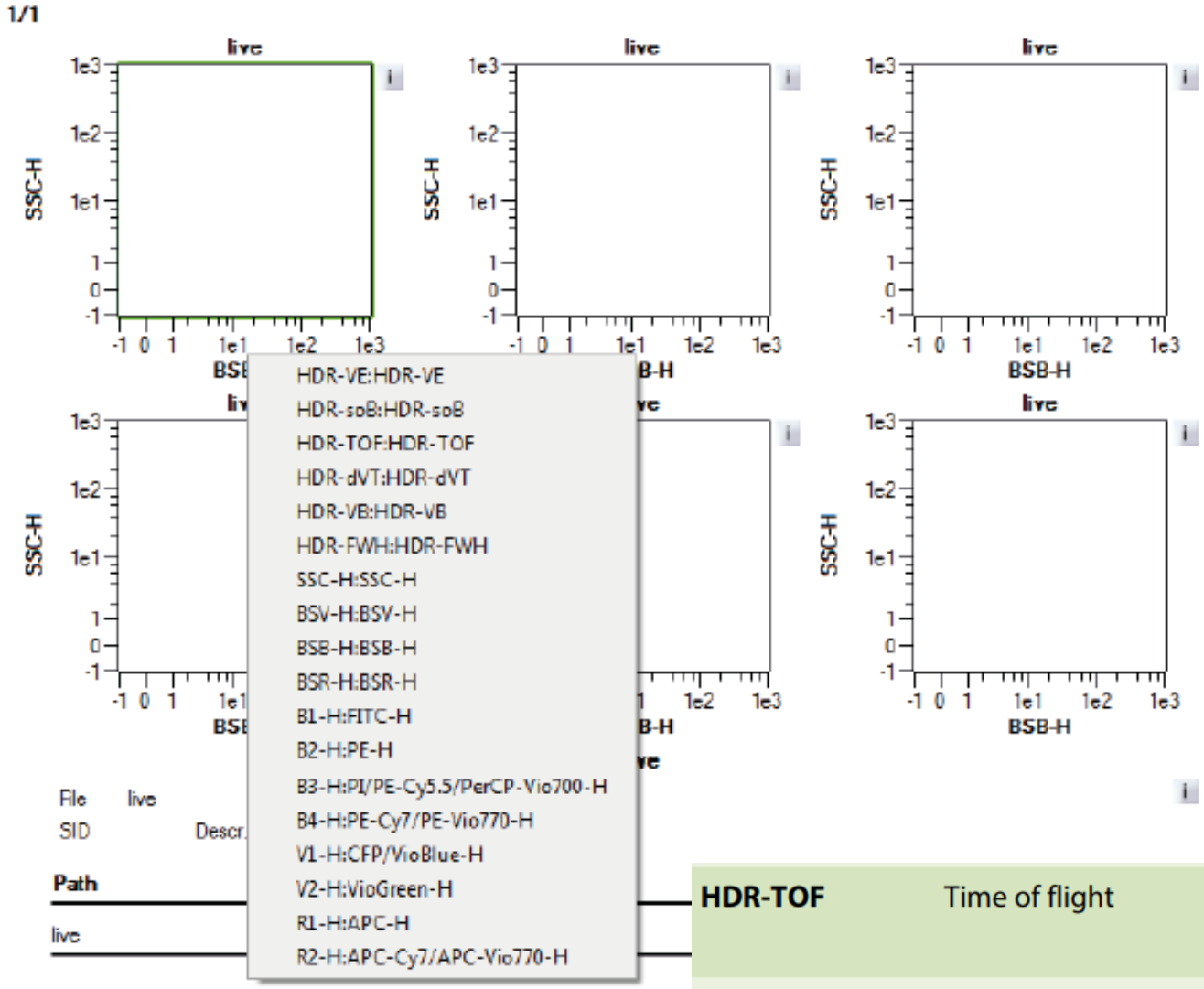
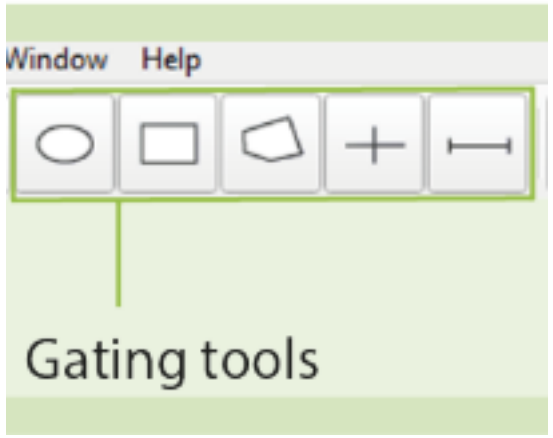


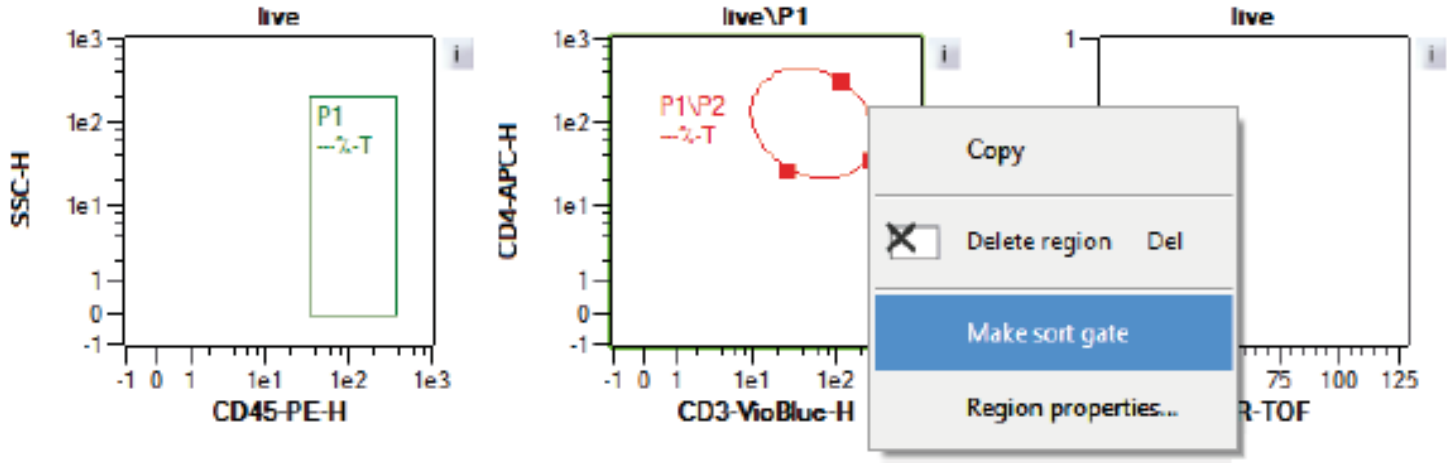
Figure 2.4: Changing the plot axis

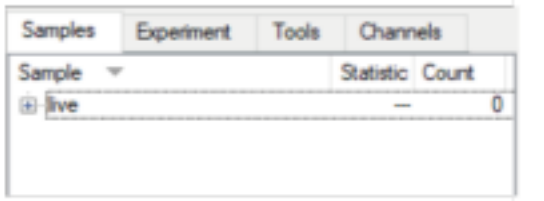
- Right click on any axis to change the parameter
- The first plot should be SSC-H by BSB-H
- Create plots BSB-H by BSR-H and BSB-H by BSV-H for adjusting scatter thresholds
- Create at least one plot for every fluorophore
- Create a plot HDR-TOF by HDR-dVT

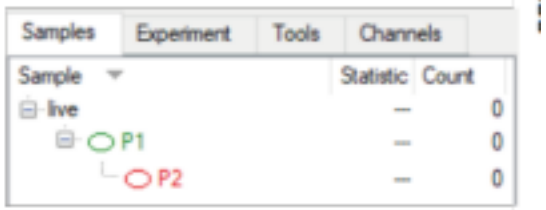
HDR-TOF	Time of flight	Time (µs) the event needs for crossing the blue and the violet laser.
HDR-dVT	Valve sync	Delta valve time : time since the last valve actuation.

Create hierarchical gating strategy and select sort gate









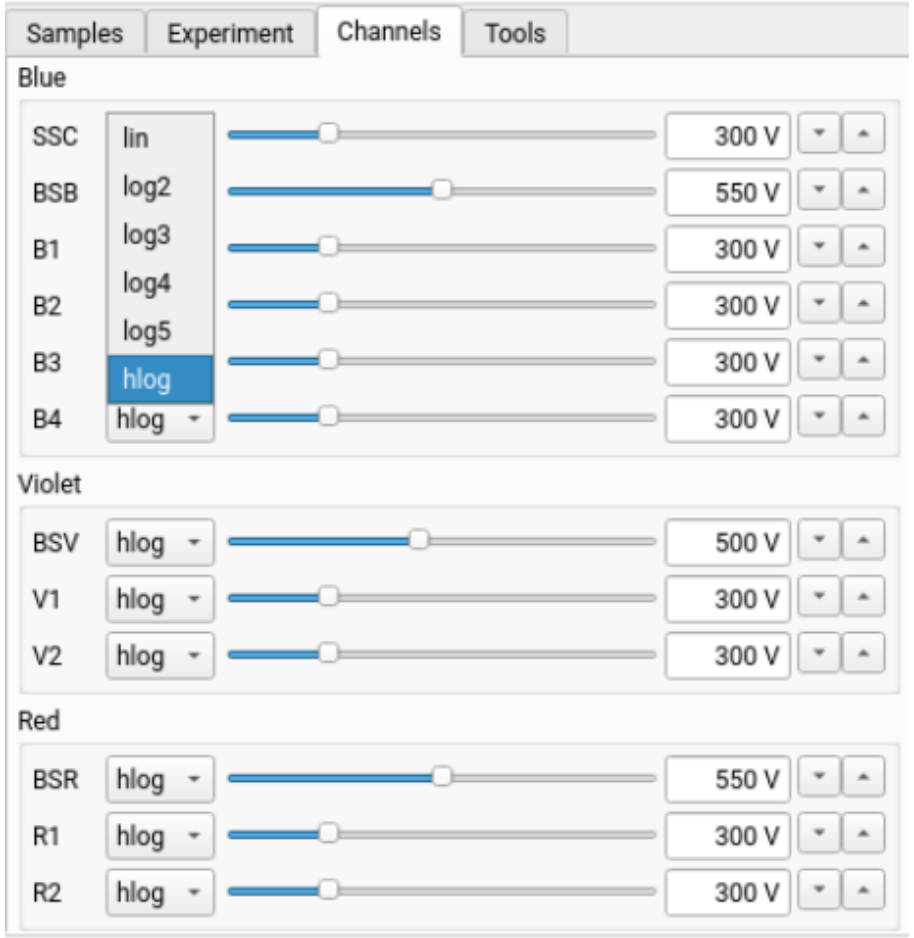
1. Draw a gate P1 on the first plot
2. Click on the title of the next plot and select P1. Only P1 events will be displayed.
3. Draw P2 gate on this plot. It will be a subset of P1
4. Click on the Plus icon next to the sample in the Samples tab to open the gating hierarchy. The hierarchy level, gate type, name and color are displayed in the hierarchy tree.
5. Right click on the appropriate gate and select “Make sort gate”

Adjust backscatter (BSC) and side scatter (SSC)

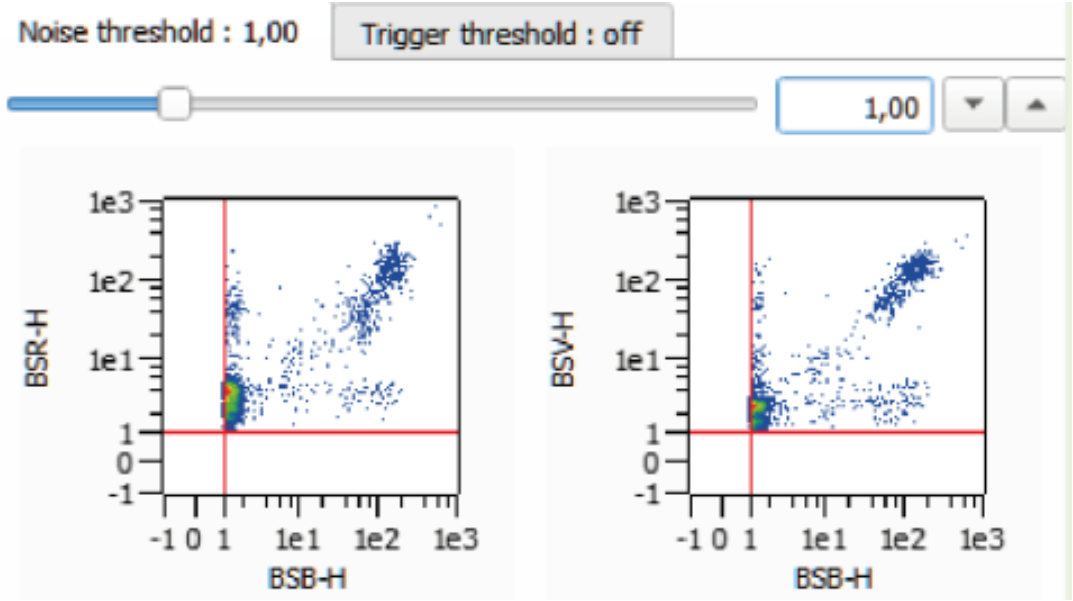
1. Click the **Start measurement** button. As events begin to accumulate, open the **Channels** tab

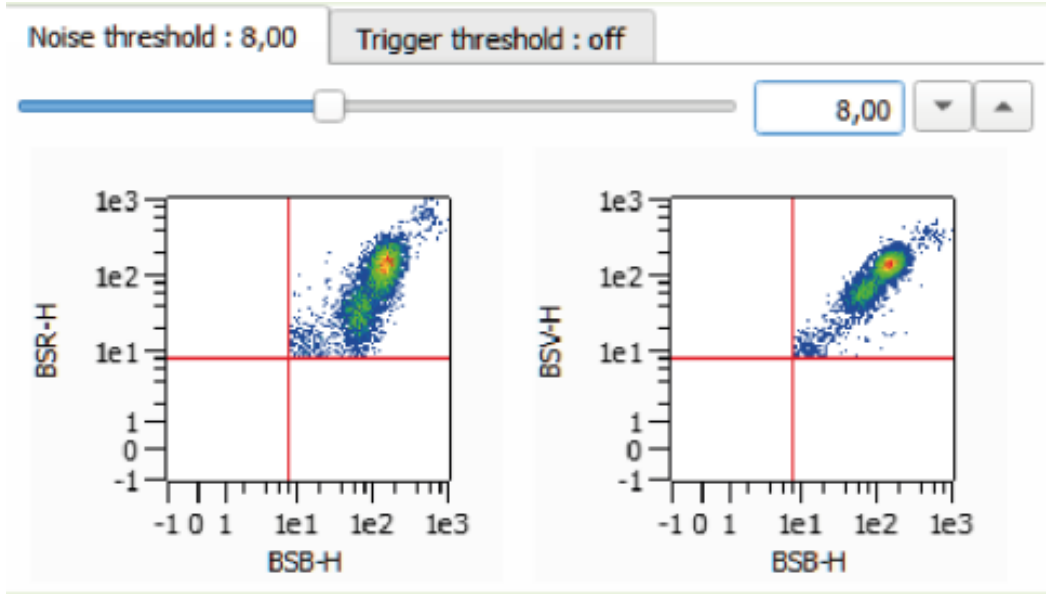
2. Adjust the voltage gains for BSC and SSC to put your cells on scale by sliding the scroll bar up or down. Gains usually need to be adjusted up. Click the **Clear** button to refresh events.

3. Adjust voltage gains on BSR, BSB and BSV so that the signals are at the top end of the scale, approximately the same value. This should be 500-550 for BSB and BSV and around 600 for BSR. Do not go over 700.



Set the noise threshold





On the Noise Threshold Tab, move the red quadrant gate to minimize detection of noise. Do this by using the slider, typing in a number or using the toggle button.

Adjust fluorescence channel gains

1. View appropriate populations on plots displaying fluorescent channels of fluorophores used in experiment
2. Under channels tab, adjust channel gains so that positive populations are bright and negative populations are on scale.
3. Note: It is not recommended to lower the PMT so that the negative population appears in the far left of the plot. This may decrease sensitivity for weakly expressed antigens.
4. Note: Avoid increasing PMT voltages above 700.

Setting the trigger threshold (optional)

1. The trigger threshold can be used to discard irrelevant events. Signals below the threshold are ignored.
2. Go to the Channels > Trigger threshold > select channel
3. Adjust slider to eliminate negative events.
4. Check the checkbox to activate the trigger threshold

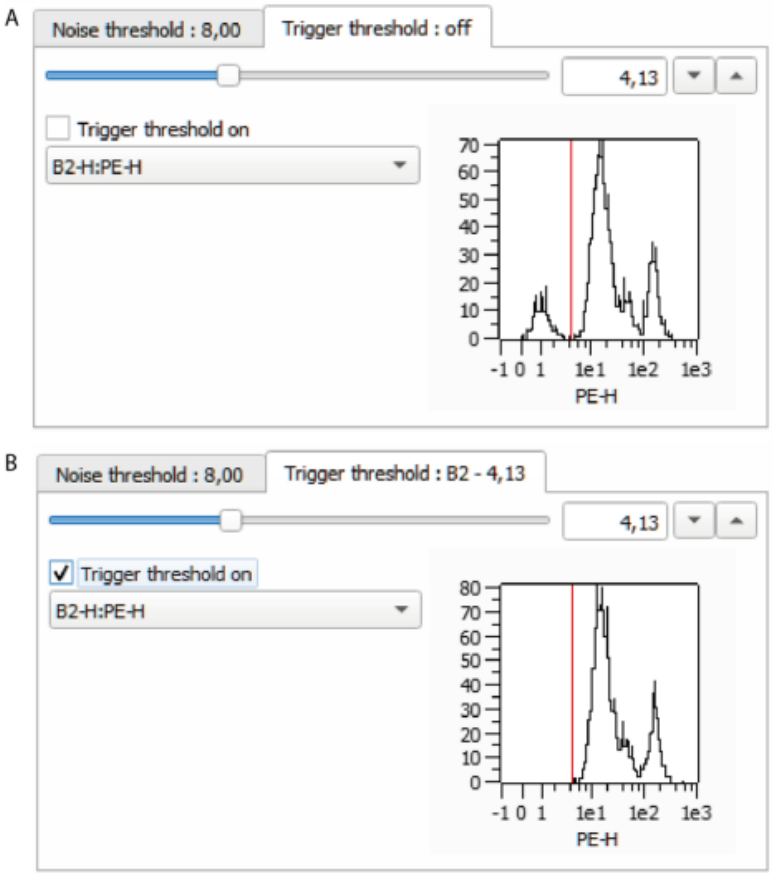


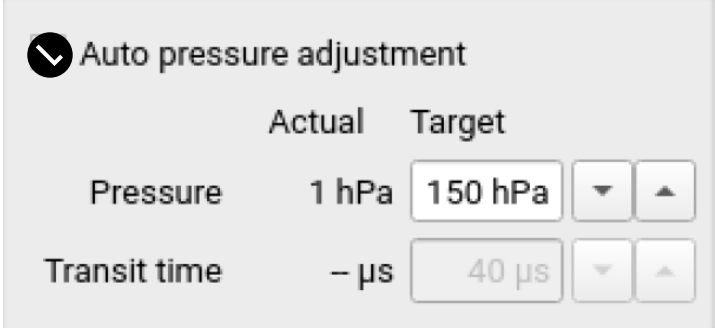
Figure 6.2: Optional trigger threshold deactivated (A) and activated (B).

Compensation

- If compensation is needed to clearly resolve your population of interest, run it after after gains are set.
- All rules of compensation still apply
- Antibody capture beads are recommended for compensation controls
- Run beads, one at a time and adjust matrix values on touch screen. Refer to the Tyto software manual page 60-63 for more detailed instructions: MACSQuantify Tyto Software Manual
- Save workspace

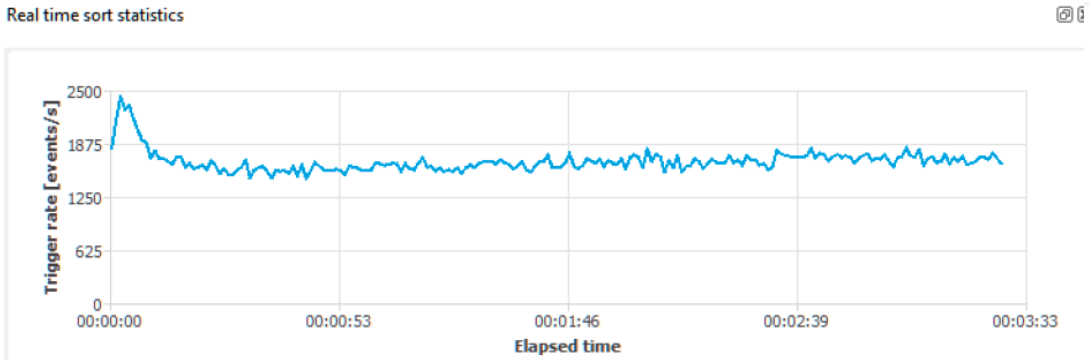
Sorting

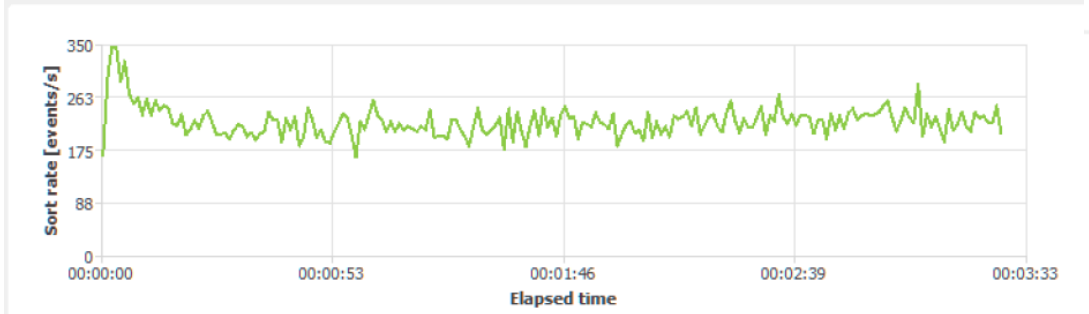
- Scan and load cartridge
- Start acquisition by clicking the **Start measurement** button
- Make sure the sort gate is selected
- Adjust sort temperature if needed
- Make sure the Auto pressure adjustment is checked in the cartridge status box
- Initiate the sort by clicking the **Sort** button

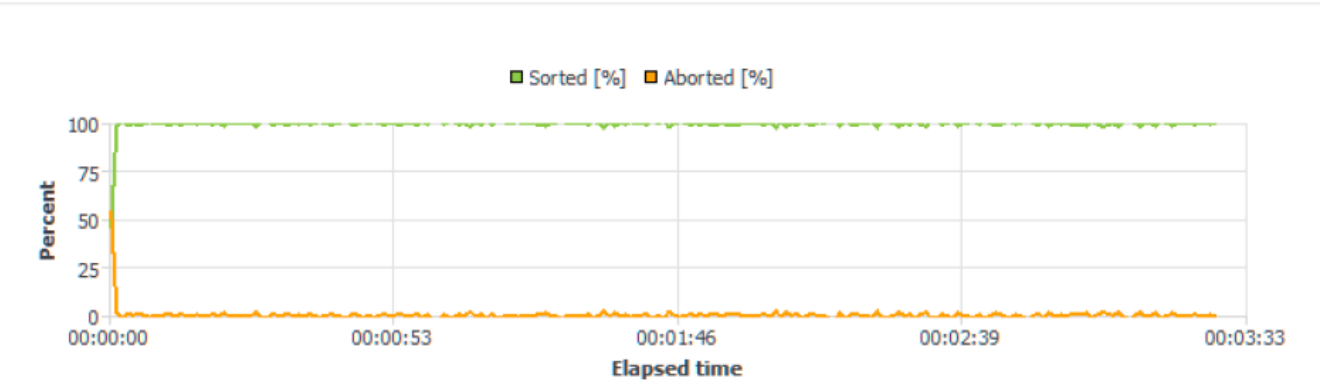


Sorting continued

- Monitor the sort in the real time sort statistics window







	Recent 10 seconds		Total time		
	Percent	Rate [events/s]	Total events	Percent	Rate [events/s]
Triggered	---	1696.4	3.469e+05	---	1666.9
Gated	13.3%	225.7	4.643e+04	13.4%	223.1
Sorted	99.5%	224.6	4.620e+04	99.7%	222.5
Aborted	0.49%	1.1	1.280e+02	0.28%	0.6

Open Report

Troubleshooting Problems

- See troubleshooting handout near Tyto
- Issues can be

Post-Sort

- The sort will stop automatically after the set sample volume is sorted or the input chamber of the cartridge is empty
- Save a Report
- Remove Cartridge
- Remove sorted fraction. Take small sample (10uL) for purity analysis on flow cytometer
- Close software and shut down computer